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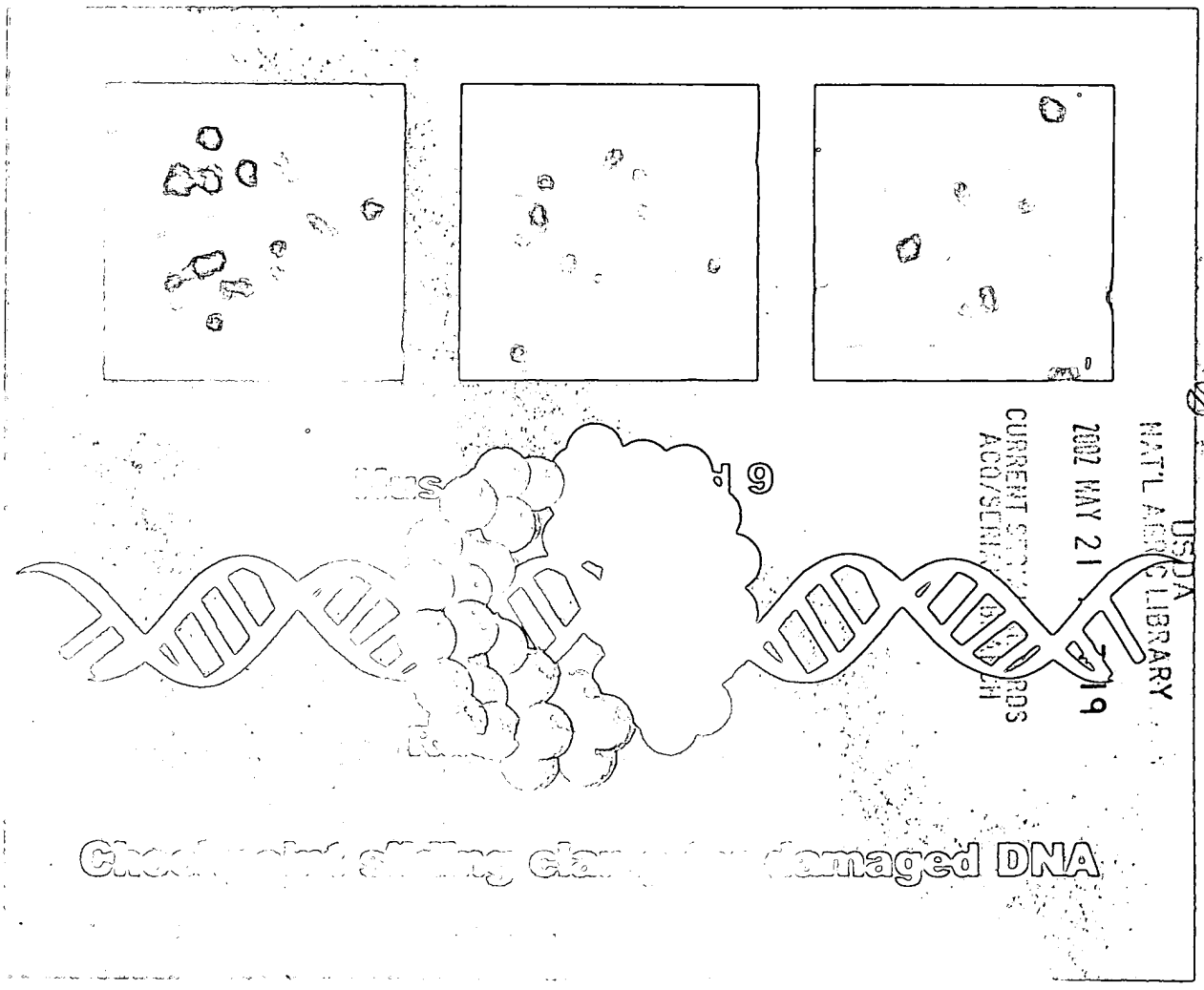

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β-Secretase Processing in the Trans-Golgi Network Preferentially Generates Truncated Amyloid Species That Accumulate in Alzheimer's Disease Brain*

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Jason T. Huse‡, Kangning Liu§, Donald S. Pijak‡, Dan Carlin‡, Virginia M.-Y. Lee§, and Robert W. Doms‡¶

From the ‡Department of Microbiology and §Center for Neurodegenerative Disease Research, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

The amyloid β (Aβ) peptide that accumulates in Alzheimer's disease brain is derived from the proteolytic processing of the amyloid precursor protein by β- and γ-secretase activities. The β-secretase enzyme β-site amyloid precursor protein-cleaving enzyme (BACE) generates the N terminus of Aβ by cleavage at either Asp[1] (β-site) or Glu[11] (β′-site), ultimately leading to the production of full-length Aβ1–40/42 or truncated Aβ11–40/42. The functional significance of this variable cleavage site specificity as well as the relative pathological impact of full-length *versus* N-terminally truncated Aβ remains largely unknown. In our analysis of BACE reactivity in cell culture, we found that the preference of the protease for either β- or β′-cleavage was strongly dependent on intracellular localization. Within the endoplasmic reticulum, β-site proteolysis predominated, whereas in the trans-Golgi network, β′-cleavage was favored. Furthermore, the contrasting cleavage site specificities of BACE were not simply due to differences in organelle pH or the oligosaccharide composition of the glycoproteins involved. Examination of post-mortem brain specimens revealed significant levels of Aβ11–40/42 within insoluble amyloid pools. Taken together, these data support an important role for β′-cleavage in the process of cerebral amyloid deposition and localize the processing event to the trans-Golgi network.

Senile plaques, lesions composed largely of aggregated amyloid β (Aβ)[1] protein, are a pathologic hallmark of Alzheimer's disease (AD) (1, 2). Aβ is derived from proteolytic processing of the type 1 membrane glycoprotein APP (3, 4), and its deposition most likely represents a crucial causative event in AD pathogenesis (5). The membrane-anchored aspartyl protease BACE acts on APP first at its β-cleavage site (6–10), generating a membrane-bound C-terminal stub (C99) whose subsequent proteolysis by a second enzyme, γ-secretase, yields Aβ. In an alternative cellular pathway precluding Aβ production, APP is initially cleaved by α-secretase activity, ultimately leading to the release of a shorter peptide known as p3 (Fig. 1*A*) (11).

Full-length Aβ encompasses a well-defined 40- or 42-amino acid residue stretch within the APP backbone (Aβ1–40 and Aβ1–42). However, in cerebral amyloid deposits, numerous N-terminally truncated variants of Aβ40 and Aβ42 (NtAβ), frequently harboring additional structural modifications, have been isolated (2, 12, 13). Whereas the functional significance of this N-terminal heterogeneity remains unclear, a variety of NtAβ species aggregate more quickly *in vitro* than their full-length counterparts (14). Whereas most types of NtAβ are assumed to arise from the proteolysis of full-length peptides after their release from cells in the central nervous system, two such variants, Aβ11–40 and Aβ11–42, are generated directly from APP by BACE proteolysis at an alternative site, termed β′, between Tyr[10] and Glu[11] of Aβ (8, 15, 16). This event initially produces a shorter C-terminal stub (C89), which then acts as a substrate for γ-secretase (Fig. 1*A*). β′-Cleavage predominates over β-cleavage in cultured neurons derived from either rats or mice (17, 18), implying that neurons in the human central nervous system may also favor this type of APP processing. In addition, Aβ11–42 has been detected as a component of amyloid in AD brain (19), further underscoring the potential significance of β′-site proteolysis in AD progression.

The discovery of BACE has provided the foundation for more comprehensive investigations into the circumstances governing both β- and β′-site cleavage. BACE appears to exist primarily in the trans-Golgi network (TGN) and the endosomal system (20, 21), although significant quantities of the glycoprotein are also present in the endoplasmic reticulum (ER) and on the cell surface (20, 22, 23). To gain a better functional understanding of BACE-APP interactions, we directed BACE to a variety of different cellular compartments by introducing discrete modifications into the protein's cytoplasmic tail. We found that the intracellular localization of BACE dramatically affected the cleavage site specificity of the enzyme. Targeting BACE to the TGN significantly enhanced β′-cleavage of APP, whereas retaining the protease in the ER resulted in primarily β-site processing. We also determined that the disparate cleavage site specificities exhibited by BACE in these two organelles are not due to differences in intracompartmental pH, oligosaccharide side chain composition, or the amount of available APP substrate. Finally, we found that NtAβ, including Aβ11–40/42, exists at levels comparable to those of full-length peptides in extracts of insoluble amyloid pools from AD brain. Thus, we provide evidence supporting a potentially crucial role for β′-cleavage in AD pathogenesis and identify the TGN as the primary cellular site where this APP processing event occurs.

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¶ To whom correspondence should be addressed: Dept. of Microbiology, University of Pennsylvania School of Medicine, 225 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104. Tel.: 215-898-0890; Fax: 215-573-2883; E-mail: doms@mail.med.upenn.edu.

[1] The abbreviations used are: Aβ, amyloid β; APP, amyloid precursor protein; BACE, β-site APP-cleaving enzyme; ER, endoplasmic reticulum; TGN, trans-Golgi network; NtAβ, N-terminally truncated Aβ; AD, Alzheimer's disease; HA, hemagglutinin; mAb, monoclonal antibody; BFA, brefeldin A; ELISA, enzyme-linked immunosorbent assay.

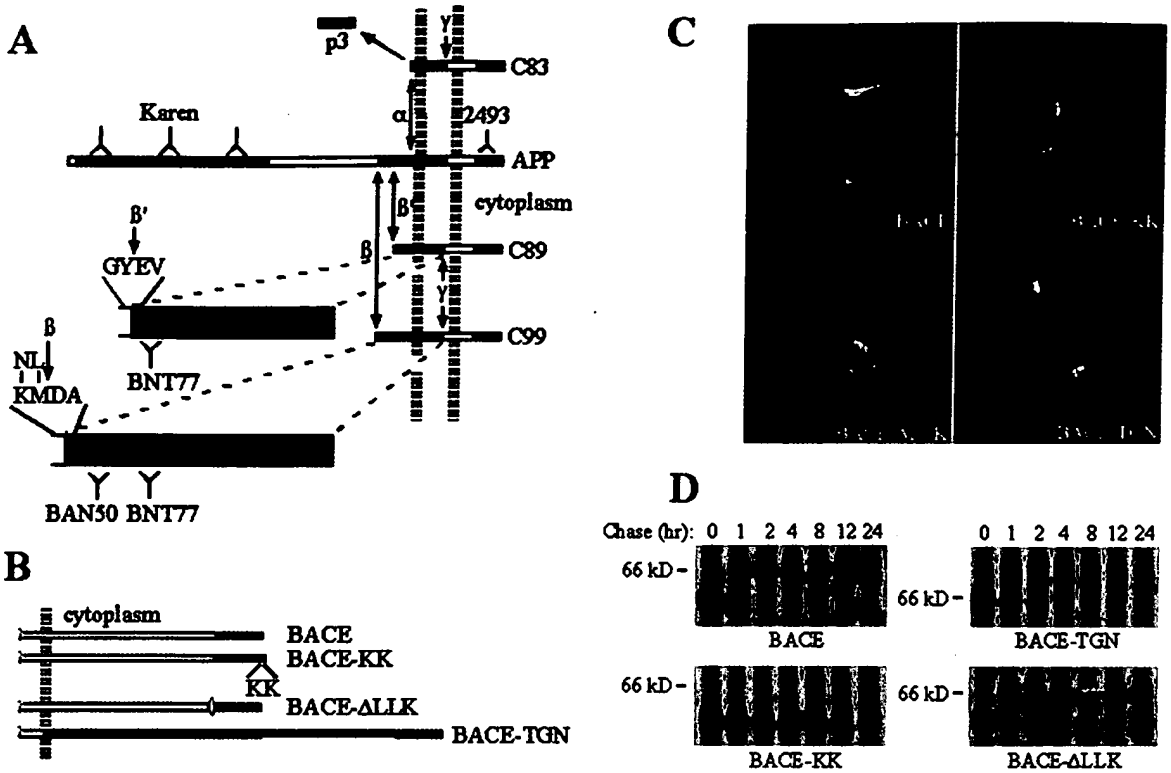

FIG. 1. **Characterization of BACE targeting mutants.** *A*, schematic illustrating APP processing. The full-length Aβ peptide region is shown in *red* with α-, β-, β'-, and γ-cleavage events indicated by *arrows*. Epitopes for the antibodies Karen (*purple*), 2493 (*black*), BAN50 (*green*), and BNT77 (*blue*) are also indicated. *B*, diagram showing the BACE constructs used in this study. *Red* denotes the cytoplasmic HA tag, whereas *green* denotes a dilysine ER retention motif. The cytoplasmic domain of furin is indicated in *blue*. *C*, immunofluorescence micrographs of HeLa cells expressing either BACE, BACE-KK, BACE-ΔLLK, or BACE-TGN. Cells were fixed and permeabilized before BACE staining with mAb HA11. *D*, 293 cells expressing either BACE, BACE-KK, BACE-ΔLLK, or BACE-TGN were radiolabeled for 30 min with [^{35}S]cysteine/methionine and chased in cold medium for the indicated amount of time. The cells were then lysed and immunoprecipitated with mAb HA11.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Expression—The derivation of BACE, BACE-KK, BACE-ΔLLK, APP, and APP-KK constructs has been described previously (20, 24). BACE-TGN was generated by oligonucleotide-based mutagenesis using the cytoplasmic tail of murine furin, a gift of Dr. Juan Bonifacino. All BACE constructs possessed a C-terminal influenza hemagglutinin (HA) tag. Stable cell lines expressing either BACE or APP constructs were obtained by plasmid selection with G418 at 0.6 mg/ml, followed in some cases by single colony subcloning.

Antibodies—HA-tagged BACE constructs were recognized with mAb HA11 (Covance). 2493, a rabbit polyclonal antisera directed against the last 40 amino acids of APP, and Karen, a goat polyclonal antisera designed against the N-terminal domain of APP, were used in the detection of APP C-terminal fragments and the full-length protein, respectively. Aβ was immunoprecipitated with 4G8, a monoclonal antibody directed against amino acids 18–24 of the peptide.

Metabolic Labeling and Pharmacological Reagents—Metabolic labeling and pulse-chase experiments were conducted as described previously (20). In some cases, additional chemical reagents were utilized at the following concentrations: brefeldin A (Sigma), 5 μg/ml; monensin (Sigma), 10 μM; NH_4Cl (Fisher), 50 mM; chloroquine (Sigma), 100 μM; and deoxymannojirimycin (Sigma), 1 mM. Endoglycosidase H digestions were performed as described previously (20).

Aβ Measurements—The sandwich ELISA technique used to quantify Aβ levels is well described (25, 26). Briefly, conditioned media were harvested after a 24-h incubation and treated with a protease inhibitor mixture (Complete; Roche Molecular Biochemicals). mAbs BAN50 and BNT77, directed against amino acids 1–10 and 11–16 of Aβ, respectively, were used as capturing antibodies. End-specific, horseradish peroxidase-conjugated mAbs BA27 (for Aβ40) and BC05 (for Aβ42) were then used for detection. Results were calibrated with standard curves using synthetic Aβ1–40 and Aβ1–42 peptides (Bachem Bioscience).

Extraction of Human Brain Tissue—0.5-g samples of cortical gray matter were separated from white matter and leptomeninges and resuspended in 2 ml of 1% Triton X-100 in 150 mM Tris, pH 7.6, 750 mM NaCl, and 2 mM EDTA with 30 strokes of a Dounce homogenizer. The lysates were then spun in a TL-100 ultracentrifuge at 40,000 rpm for 1 h at 4 °C. After extraction of the supernatants, the insoluble pellets were resuspended in 1 ml of 70% formic acid with brief sonication before a second high-speed centrifugation. The supernatants were extracted, and the remaining material was discarded. Formic acid extractions were then lyophilized and resuspended in 100 μl of 70% formic acid, followed by neutralization in 1.9 ml of 1 M Tris base.

Mass Spectrometry—Conditioned media samples and brain extracts, both detergent- and formic acid-soluble, were immunoprecipitated with 4G8. Immunoprecipitates were then eluted in a minimum volume of matrix solution (α-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile and 0.1% trifluoroacetic acid) and analyzed in a Micromass Tof Spec2E mass spectrometer. Results were expressed as a percentage of the largest peak.

RESULTS

The Cellular Localization of BACE Affects APP Cleavage—BACE and APP have the potential to interact in several distinct cellular compartments. To determine the extent and efficiency of β- and β'-site processing at selected points within the cell, BACE mutants targeted to different organelles were generated (Fig. 1*B*). The addition of two lysine residues to the −3 and −4 positions on the BACE cytoplasmic tail (BACE-KK) effectively retains the protein in the ER, whereas elimination of a C-terminal dileucine motif (BACE-ΔLLK) leads to accumulation on the cell surface (20). A BACE chimera was also generated whose cytoplasmic tail was replaced with the intracellular domain of murine furin (BACE-TGN), a modification that has been shown to effectively target type I membrane proteins to the TGN (27). Immunostaining of HeLa cells expressing BACE targeting mutants confirmed their altered localization patterns relative to the wild type protein (Fig. 1*C*), and metabolic pulse-chase analysis showed that all species exhibited similar turnover rates and, with the exception of ER-retained BACE-KK, matured efficiently with wild type kinetics (Fig. 1*D*).

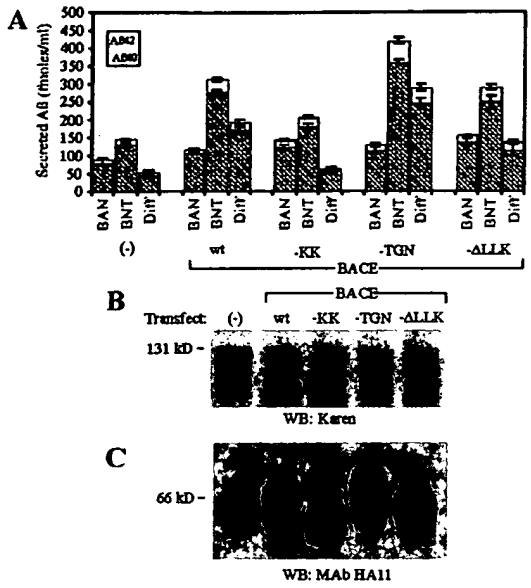

FIG. 2. **The impact of BACE localization on Aβ production.** 293 cells stably expressing wild type APP were transfected with BACE targeting mutants. Secreted Aβ from conditioned media was then quantified by sandwich ELISA using either BAN50 or BNT77 as capturing antibody (*A*; see "Experimental Procedures"). The third bar in each set (*Diff*) indicates the difference between the measured levels of BNT77- and BAN50-detectable Aβ. Equal expression levels of APP and BACE were verified by Western blot of cell lysates with either Karen (*B*) or mAb HA11 (*C*), respectively.

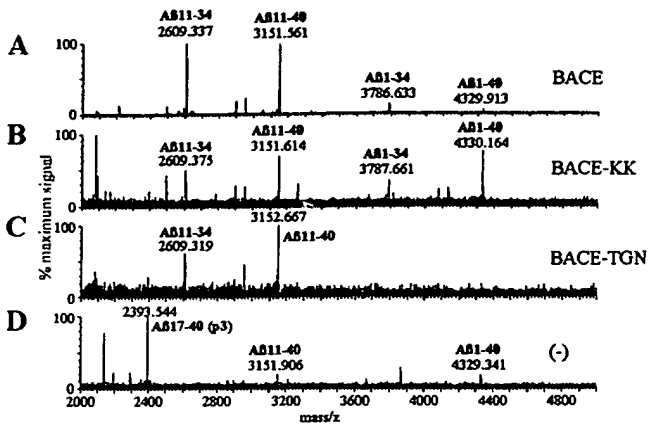

FIG. 3. **Aβ11-X species represents the primary secreted NtAβ variants.** 293 cells stably expressing either BACE (*A*), BACE-KK (*B*), or BACE-TGN (*C*) and parental (−) 293 cells (*D*) were transfected with APP. Conditioned media collected after a 24-h incubation were then immunoprecipitated with 4G8 and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Peak size and assignment are shown.

To assess the impact of BACE localization on the production of Aβ, we transfected BACE targeting mutants into 293 cells stably expressing wild type APP. In each experiment, the relative levels of APP and each BACE targeting mutant were determined to be equivalent by Western blot (Fig. 2, *B* and *C*). Conditioned media collected after a 24-h incubation were then analyzed for Aβ by sandwich ELISA using one of two monoclonal capturing antibodies with distinct, nonoverlapping epitopes (Fig. 2*A*; see "Experimental Procedures"). BAN50 captures primarily Aβ1–40 and Aβ1–42, whereas BNT77 detects N-terminally truncated species as well as full-length peptides. We found that expression of BACE resulted in a modest increase in BAN50-detectable Aβ coupled with a more substantial rise in the level of peptide captured by BNT77, indicating that significant amounts of NtAβ were being generated in addition to full-length peptide (Fig. 2*A*, *BAN*, *BNT*, and *Diff*). BACE-ΔLLK had similar effects. By contrast, retaining BACE in the ER led to a much smaller difference between the levels of BAN50- and BNT77-detectable Aβ, whereas targeting BACE to the TGN increased the size of this discrepancy. Thus, BACE appeared to exhibit different cleavage site specificities depending on its precise localization within the secretory pathway. Expression of BACE in the ER resulted primarily in the production of Aβ1–40 and Aβ1–42, whereas targeting the protease to the TGN led to enhanced secretion of N-terminally truncated peptides.

We further investigated the precise identity of these NtAβ species by immunoprecipitation-mass spectrometry. 293 cells stably expressing comparable and moderate levels of BACE, BACE-KK, or BACE-TGN along with parental 293 cells were transiently transfected with wild type APP. Conditioned media samples collected after a 24-h incubation were then immunoprecipitated with 4G8, a monoclonal antibody directed against amino acid residues 17–24 of Aβ. Whereas this technique is not entirely quantitative, it does allow for identification of the major amyloid constituents present. We found by this analysis that Aβ1–40, Aβ11–40, Aβ1–34, and Aβ11–34 were the primary detectable species in conditioned media taken from cells coexpressing BACE and wild type APP, consistent with our earlier findings (Fig. 3*A*) (28). Previous studies have reported the presence of AβX-34 species in extracts of cerebrovascular amyloid (29) and demonstrated that cleavage at position 34 occurs readily in neurons expressing APP (30). The same Aβ variants seen in the context of wild type BACE were also observed in parallel experiments with either BACE-KK or BACE-TGN. However, for BACE-KK, more Aβ1-X was recovered relative to Aβ11-X, whereas for BACE-TGN, Aβ11-X peptides predominated, yielding similar spectra to those obtained in the context of wild type BACE (Fig. 3, *B* and *C*). Media taken from parental 293 cells transfected with APP yielded primarily Aβ17–40 (p3), with lower levels of both Aβ1–40 and Aβ11–40 (Fig. 3*D*). In each case, Aβ species beginning at Glu^{11} represented the only NtAβ variants recovered. Levels of Aβ42 peptides were consistently below our detection limits. These studies further support the conclusion that targeting BACE to different intracellular sites alters the levels of full-length and truncated Aβ ultimately secreted by cultured cells. They also demonstrate that the major NtAβ species generated are the products of β′-cleavage by BACE.

β′-Cleavage of APP Occurs Most Readily in the TGN—Our sandwich ELISA results led us to conclude that the intracellular localization of BACE might have profound effects on the efficiency of β- and/or β′-site proteolysis. To verify this possibility, we investigated the impact of BACE targeting mutants on the generation of APP C-terminal fragments, the immediate products of β-secretase cleavage. Unlike the measurement of secreted Aβ levels, this approach employed a single antisera (2493) in the analysis of all APP C-terminal fragments (Fig. 1*A*). 293 cells stably expressing BACE, BACE-KK, or BACE-TGN along with parental 293 cells were transiently transfected with wild type APP. After metabolic labeling for 1 h, the cells were lysed, and APP C-terminal fragments were immunoprecipitated (Fig. 4*A*).

As expected, expression of APP in parental 293 cells resulted in the almost exclusive production of α-secretase-derived C83, indicating that endogenous β-secretase activity in this cell type is quite low (Fig. 4*A*, *lane 1*). By contrast, expression of exogenous BACE led to high levels of C99 and especially C89 in addition to C83, consistent with earlier studies (Fig. 4*A*, *lane 2*) (15, 16). Retaining BACE in the ER resulted in the generation of C99 in excess of C89 (Fig. 4*A*, *lane 3*), whereas targeting

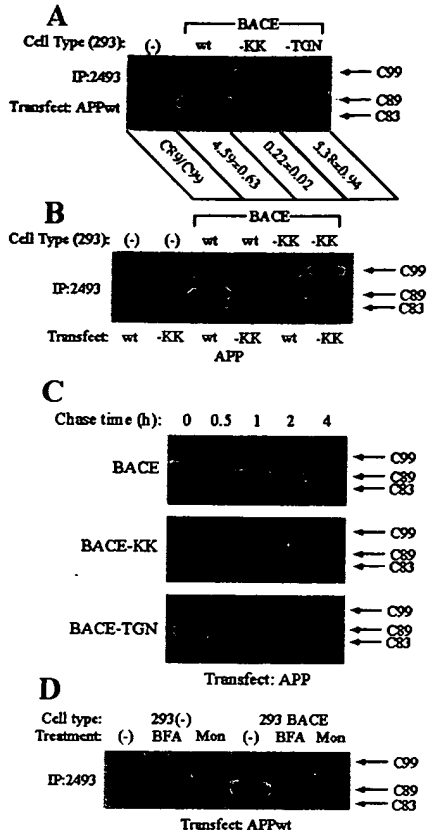

FIG. 4. **Cellular localization determines BACE cleavage site specificity.** *A*, 293 stable cell lines expressing either BACE, BACE-KK, or BACE-TGN and parental 293 cells (−) were transfected with wild type APP, radiolabeled, lysed, and immunoprecipitated with 2493. The ratios of C89/C99 band intensities are shown. *B*, 293 stable cell lines expressing BACE or BACE-KK were transfected with APP or APP-KK. Analysis of APP C-terminal fragments revealed that high levels of APP and BACE in the same cellular compartment are insufficient to promote β′-cleavage. *C*, 293 stable cell lines expressing either BACE, BACE-KK, or BACE-TGN were transfected with APP, radiolabeled for 1 h with [^{35}S]cysteine/methionine, and chased in cold medium for the indicated amount of time. The cells were then lysed and immunoprecipitated with 2493. *D*, parental 293 cells and 293 cells stably expressing BACE were transfected with wild type APP before a 1-h pretreatment and 1-h radiolabeling in the presence of either BFA, monensin (*Mon*), or vehicle alone (−). The cells were then lysed and immunoprecipitated with 2493.

BACE to the TGN had the reverse effect, leading to the almost exclusive production of C89 (Fig. 4*A*, *lane 4*; see quantification). Finally, APP C-terminal fragment production in the context of BACE-ΔLLK did not differ significantly from that observed for wild type BACE (data not shown). Thus, restricting BACE to the ER resulted primarily in β-site cleavage, whereas allowing BACE to accumulate in the TGN up-regulated β′-proteolysis. These results are consistent with our sandwich ELISA and mass spectrometry results and, furthermore, demonstrate that BACE processing of APP determines the relative levels of full-length and truncated Aβ ultimately secreted by cells.

Previous studies have suggested that β′-cleavage may simply be a nonphysiological artifact of overexpression (16). In light of these reports, one could argue that heightened β′-cleavage in the TGN may simply be due to the accumulation of BACE protein in a cellular compartment containing high levels of APP substrate. To address this possibility, we transfected our BACE-KK-expressing cell line with either wild type APP or ER-retained APP-KK (Fig. 4*B*). We reasoned that if high levels of BACE and APP in the same cellular compartment promote β′-proteolysis, overexpressing APP in the ER should result in an elevation of the C89/C99 ratio. Analysis of C-terminal fragment production revealed that retention of APP in the ER along with BACE, while generally increasing the levels of APP C-terminal fragments, did not result in a relative enhancement of β′-site cleavage (Fig. 4*B*, *lanes 5* and *6*). Thus, the simple overexpression of BACE and APP in the same cellular compartment is insufficient to promote β′-cleavage. We also considered whether C89 and C99 might be degraded at different rates within different cellular compartments, a possibility that could explain the contrasting levels of APP C-terminal fragments in our organelle targeting studies. 293 cells coexpressing wild type APP with either BACE, BACE-KK, or BACE-TGN were subjected to metabolic pulse-chase analysis with 2493 followed by SDS-PAGE resolution of immunoprecipitated C-terminal fragments. These experiments revealed that C89 and C99 were degraded at similar rates regardless of the site of their generation (Fig. 4*C*).

To more accurately define the intracellular site(s) where β′-processing occurs, we analyzed BACE reactivity in the presence of pharmacological reagents that block protein transport at different points within the secretory pathway. Brefeldin A (BFA) halts the transport of proteins at the level of the ER (31), whereas the ionophore monensin arrests protein maturation at defined points within the Golgi apparatus in a cell type-specific manner (32). In our 293 stable cell lines, we found by analysis of oligosaccharide side chain maturation that this block occurred between the medial and trans-Golgi (data not shown). Cells expressing BACE were then transfected with wild type APP and pretreated with either BFA, monensin, or vehicle alone for 1 h before metabolic labeling, lysis, and immunoprecipitation with 2493. We found that both BFA and monensin significantly hampered β-secretase processing of APP (Fig. 4*D*). However, β- and β′-cleavage were not affected equally in either case. Monensin treatment primarily impaired the generation of C89 but only slightly decreased C99 production (Fig. 4*D*, *lanes 4* and *6*). BFA completely eliminated β′-processing, whereas the generation of C99 was maintained, albeit at a lower level (Fig. 4*D*, *lanes 4* and *5*). These results indicated that transport through the late Golgi is required for efficient β′-cleavage of APP, again implicating the TGN as a preferred site for β′-processing.

Organelle-specific Cleavage of APP by BACE Is Not due to Differences in pH or Oligosaccharide Side Chain Composition—Differential cleavage of APP by BACE in the ER and TGN could simply result from the contrasting environments of these two cellular compartments. We first investigated whether the difference in pH between the ER and TGN (the ER exhibits a neutral pH, whereas the TGN is mildly acidic (pH 5.9–6.5) (33)) determines the relative efficiencies of β- and β′-proteolysis. To do this, we analyzed APP C-terminal fragment production in the presence of NH_4Cl and chloroquine, weak bases that neutralize the pH of acidic organelles. 293 cells coexpressing APP with either BACE or BACE-TGN were left untreated or incubated with either NH_4Cl or chloroquine for 1 h before radiolabeling, lysis, and immunoprecipitation with 2493. We found that in the presence of weak base, β-secretase processing for both BACE and BACE-TGN was only slightly altered, such that C99 production increased incrementally, concomitant with a small decrease in C89 levels (Fig. 5, *A* and *B*). By contrast, both NH_4Cl and chloroquine completely inhibited the endoproteolysis of cathepsin D, a processing event that requires an acidic microenvironment, indicating that the reagents themselves functioned appropriately (data not shown). Thus, neutralizing the pH of the TGN to a level resembling that of the ER failed to dramatically affect β-secretase processing of APP, a finding that suggests pH plays only a minor role in directing BACE toward either β- or β′-cleavage.

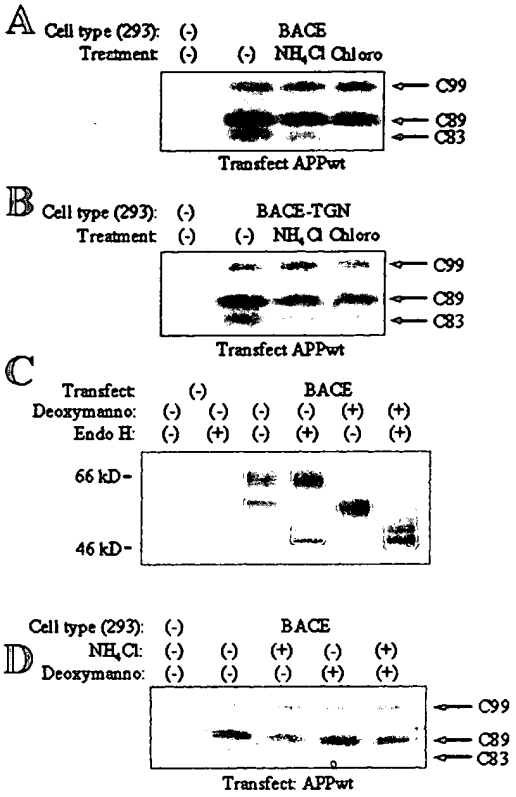

FIG. 5. **BACE cleavage site specificity cannot be attributed entirely to organelle pH or oligosaccharide side chain composition.** Parental 293 cells (−) and 293 cells stably expressing either BACE (*A*) or BACE-TGN (*B*) were transfected with wild type APP. The cells were radiolabeled, lysed, and immunoprecipitated with 2493. Where indicated, the cells were pretreatcd for 1 h and radiolabeled in the presence of either NH_4Cl or chloroquine (*Chloro*). *C*, 293 cells expressing BACE were left untreated or incubated in the presence of deoxymannojirimycin (*Deoxymanno*) overnight. The cells were subsequently radiolabeled for 2.5 h (in the presence of deoxymannojirimycin where indicated), lysed, and immunoprecipitated with mAb HA11. Immunoprecipitates were then left untreated or digested with endoglycosidase H. *D*, 293 cells stably expressing BACE were transfected with wild type APP and either left untreated or incubated in the presence of deoxymannojirimycin overnight to ensure that the majority of normally glycosylated BACE was degraded. The cells were also pretreated for 1 h with NH_4Cl where indicated. Radiolabeling (in the presence of drugs as shown), lysis, and immunoprecipitation with 2493 revealed that deoxymannojirimycin has little effect on β-secretase processing.

Both BACE and APP contain *N*-linked carbohydrate side chains that exist in an immature, "high mannose" form in the ER before processing in the Golgi and TGN to more complex variants. Thus, the structures of BACE and APP differ to at least some extent in the ER and the TGN. To address the potential role of oligosaccharide side chain maturation in β-secretase processing, we utilized deoxymannojirimycin, a Golgi mannosidase 1 inhibitor that arrests the development of *N*-linked oligosaccharides at the high mannose stage, characteristic of ER residence. In the presence of deoxymannojirimycin, BACE remained sensitive to endoglycosidase H, indicating that the *N*-linked side chains of the protein were indeed in an immature state (Fig. 5*C*). However, treatment of 293 cells coexpressing BACE and APP with deoxymannojirimycin did not alter the production of APP C-terminal fragments, nor were the effects of NH_4Cl on β-secretase processing synergistically enhanced by the reagent (Fig. 5*D*). Finally, as for many other (but not all) proteins, deoxymannojirimycin was found to have no impact on the cellular distribution of either APP or BACE by immunofluorescence microscopy (data not shown) (34). These results demonstrated that the oligosaccharide side chain compositions of BACE and APP have little effect on the determination of β-secretase cleavage site specificity in our cell culture system.

β′-Cleavage Products Aβ11–40 and Aβ11–42 Accumulate in Alzheimer's Disease Brain—Our results indicate that β′-cleavage of APP is a specific, intracellular processing event that occurs most efficiently in the TGN. To determine whether the β′-site cleavage products Aβ11–40/42 represent a significant fraction of total Aβ in the human central nervous system, we investigated the composition of insoluble amyloid pools extracted from post-mortem brain tissue. Gray matter from the mid-frontal cortices of four individuals with confirmed AD and three normal controls was homogenized in a high-salt, Triton X-100 lysis buffer. The remaining material was then extracted in 70% formic acid, a treatment that has been shown to effectively recover large amounts of otherwise insoluble Aβ both *in vitro* and *in vivo* (30, 35). The levels of Aβ in this fraction, especially Aβ42, have been demonstrated to be substantially higher in AD patients than in unaffected individuals, underscoring the potential importance of this insoluble pool in AD pathogenesis (35). However, the relative solubilities of full-length Aβ *versus* the more fibrillogenic NtAβ variants in formic acid have not been extensively studied.

We investigated the composition of Aβ in both soluble and insoluble fractions by immunoprecipitation-mass spectrometry with 4G8. The levels of Aβ recovered by detergent extraction were generally too low to effectively detect (data not shown). However, spectrometric analysis of formic acid fractions from AD brains revealed several peaks corresponding to the calculated masses of full-length Aβ1–40/42 along with several different N-terminally truncated variants (Fig. 6*A*). Aβ11–42 was invariably present, and in three of four cases, both Aβ11–40 and Aβ11–42 were observed at significant levels comparable to those of Aβ1–42 after summation of their free glutamate and pyroglutamate isoforms. In addition, Aβ11–42 represented the primary detectable N-terminally truncated Aβ42 species. Mass spectrometry of formic acid extractions derived from normal brain demonstrated no significant peaks (Fig. 6*B*). Thus, our analysis revealed that the pool of insoluble Aβ, which exists at high levels in AD brain, includes Aβ11–40 and Aβ11–42 as major components.

DISCUSSION

The identification of BACE as the β-secretase associated with AD has provided an attractive molecular target for pharmaceutical intervention, especially given the relatively subtle phenotype exhibited by BACE knockout mice (18, 36). Our investigations using organelle targeting mutants demonstrated that BACE effectively processes APP in several different cellular compartments and, more interestingly, that the location of the protease within the cell dramatically affects its cleavage site specificity. Analysis of both secreted Aβ and C-terminal fragment precursors revealed that ER-retained BACE-KK cleaved APP primarily at its β-site, whereas β′-proteolysis predominated in the context of BACE-TGN. BACE-ΔLLK, by contrast, did not differ noticeably from BACE-HA in its APP processing characteristics, a fact most likely due to similarities in cellular distribution of this BACE variant to that of the wild type protein. Despite altered recycling kinetics that promote its accumulation on the cell surface, BACE-ΔLLK does undergo internalization from the plasma membrane at bulk rate and most likely cycles through the same intracellular compartments (*i.e.* endosomes and the TGN) as its wild type counterpart (20). Blocking protein transport prior to the late Golgi with either BFA or monensin severely reduced β′-site proteolysis, with more subtle effects on β-cleavage. This find-

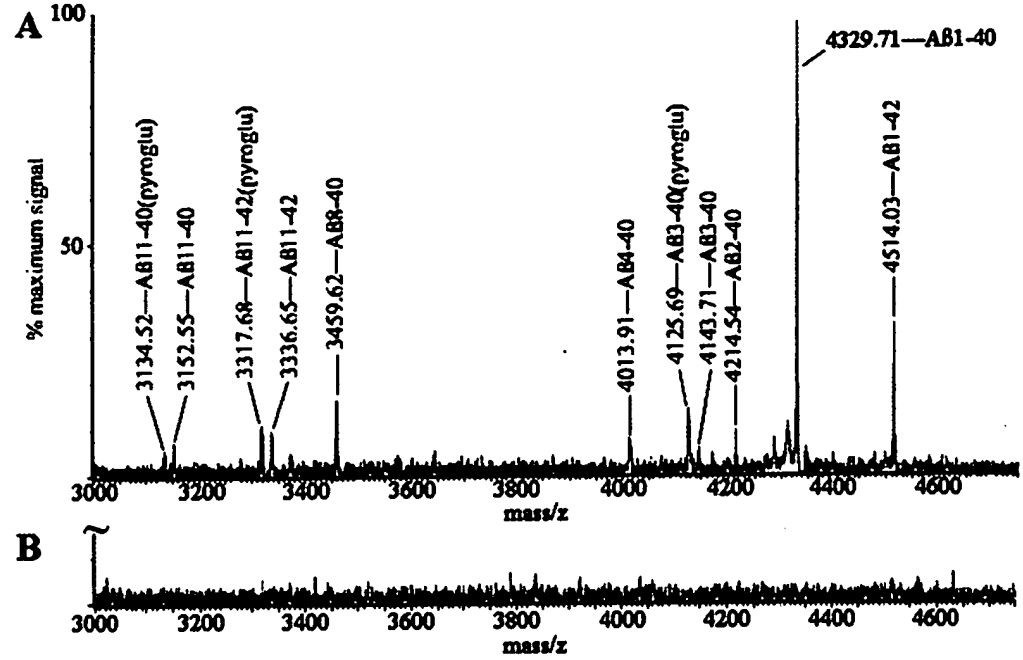

FIG. 6. **The composition of insoluble Aβ extracted from AD (*A*) and normal control (*B*) brains with 70% formic acid was analyzed by immunoprecipitation-mass spectrometry (see "Experimental Procedures").** Peak size and assignment are shown. N-terminal pyroglutamate species are also indicated (*pyroglu*).

ing, in concert with our BACE targeting studies, identifies the TGN as a major site for β′-cleavage within the cell.

The efficiency of β′-cleavage as judged by direct analysis of APP C-terminal fragments was generally higher than that observed by sandwich ELISA (compare Figs. 2*A* and 4*A*). Whereas this discrepancy may reflect a reduced ability of γ-secretase to effectively cleave C89 relative to C99, the nearly identical degradation rates of the different C-terminal fragments observed by pulse-chase analysis argue against this possibility. A more likely explanation is that the generally higher levels of APP and BACE expression required for consistent visualization of C89 and C99 lead to increased processing at Glu^{11}. Previous work has, in fact, demonstrated that β′-cleavage efficiency increases with BACE overexpression, implying that processing at this alternative site may simply be the result of nonphysiologically high levels of BACE and APP (16). The bulk of our findings, however, are not consistent with this possibility. Targeting equal levels of BACE protein to different cellular sites dramatically affected the extent to which β- or β′-site proteolysis was preferred, demonstrating that environmental conditions play an important role in the determination of cleavage site specificity. In addition, overexpression of BACE in the ER led primarily to β-site processing, a tendency that was not altered even when high levels of APP were retained in the same organelle. The observation that β′-cleavage is more pronounced with increasing APP and BACE levels may therefore reflect differences in the trafficking of the proteins rather than their absolute amounts. For instance, overexpression of BACE and APP may result in their accumulation in cellular compartments such as the TGN where β′-cleavage is favored.

We and others have shown that BACE can effectively act on C99 as well as full-length APP at the β′-site (16, 28), and one or both of these mechanisms could be enhanced in the TGN. However, the precise environmental characteristics responsible for BACE cleavage site specificity within different cellular compartments remain unclear. Our data demonstrated that the preference of BACE for either β- or β′-cleavage does not depend solely on organelle pH or the oligosaccharide side chain composition of resident and itinerant glycoproteins. Nevertheless, other possibilities exist. For instance, the presence or absence of the BACE propeptide, which is removed only after export from the ER (20, 22, 23), could influence the cleavage site specificity of the protease. *In vitro* studies have shown that the propeptide region of BACE serves more in the facilitation of proper folding than in the regulation of β-site cleavage (37). The impact of the BACE pro-domain on β′-site proteolysis, however, has not been directly addressed.

The cleavage site specificity of BACE could also be influenced by other associated proteins that could promote either β- or β′-site proteolysis in different organelles. It is not entirely surprising that BACE could function in the context of a multicomponent catalytic complex, even considering the current lack of evidence that the protease forms oligomeric structures (20). It should be noted that most evidence suggests that γ-secretase exists as a large protein complex whose functional components include the presenilins and the more recently identified glycoprotein nicastrin (38–41). Whereas *in vitro* studies have demonstrated optimal BACE activity only within a relatively tight pH range (pH 4.0–4.5) (8, 10), we have shown that BACE readily acts on APP within the ER, a neutral cellular compartment, and the mildly acidic TGN. These findings imply that other unidentified proteins or environmental factors may serve to facilitate BACE activity and determine cleavage site specificity.

The fact that BACE cleaves at two distinct sites on APP could have a considerable impact on amyloid deposition in the central nervous system. NtAβ species, including Aβ11–40/42, exhibit fibrillogenic characteristics *in vitro* consistent with increased aggressiveness and neurotoxicity relative to their full-length counterparts (14). Whether NtAβ variants predominate in AD, however, has not been definitively established. Our mass spectrometry results indicate that significant amounts of Aβ11–40/42 exist in insoluble extracts derived from AD brain, and Aβ11–42 appears to represent the primary truncated component of the large, well-documented pool of Aβ42 present in AD brain (35). Furthermore, we cannot exclude the possibility that Aβ11–40/42, along with other NtAβ species, are less ef-

fectively solubilized by formic acid than full-length peptide, implying the existence of even higher levels of truncated material. Whereas earlier studies have isolated several distinct NtAβ variants from cerebral amyloid depositions (2, 12, 19), none have consistently demonstrated the presence of both Aβ11–40 and Aβ11–42. Investigations using mouse models expressing both APP and BACE should provide a more thorough analysis of the importance of β- *versus* β′-cleavage in the various stages of amyloid deposition and toxicity.

In conclusion, we have demonstrated that an alternative β-secretase processing event in the TGN may have a significant impact on neurodegeneration in AD, warranting its close examination. Our finding that the cleavage site specificity of BACE is dependent on intracellular localization implies that distinct molecular mechanisms most likely govern β- and β′-cleavage and, furthermore, that different therapeutic strategies could be developed to address each processing event independently.

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